

Remarks

Applicants have amended the specification to provide priority application information and information regarding the publication in English under PCT Article 21(2) of the PCT application of which the above-identified application is a U.S. national stage application. applicants also have amended the specification to add references to SEQ ID NOS of the sequence listing filed herewith. No new matter has been added.

Applicants have also corrected two typographical errors on page 19, line 11 of the specification. The identifying name of the two primers was in error. The identical two primers were correctly referred to on page 19 lines 2 and 4, and their subsequent incorrect identification was the result of a typographical error. The error has been corrected in the substitute paragraph. No new matter has been added.

In addition, a typographical error was made in the labeling of the sequences in Figure 8. The identifying names of the second and third sequences (as NAALAD L and NAALAD II) were inadvertently reversed. The correct identity of the two sequences is supported by the sequences and names in Figure 6, which contains the full sequence, of which the sequences in Figure 8 are fragments. The identifying names were inadvertently mistyped during creation of Figure 8 and have been corrected in the substitute Figure 8. No new matter has been added.

Respectfully submitted,



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New Section:**Related Applications**

This application is a national stage filing under 35 U.S.C. §371 of PCT International application PCT/GB99/02241, filed July 14, 1999, which was published under PCT Article 21(2) in English. Foreign priority benefits are claimed under 35 U.S.C. §119(a)-(d) or 35 U.S.C. §365(b) of Great Britain application number GB 9815284.6, filed July 14, 1998.

Marked-up Paragraphs as Amended:

Please substitute the following paragraph for the paragraph beginning on page 11, line 20:
Figure 1: is an illustration of the nucleotide and amino acid sequences of human NAALAD-ase L. The nucleotide (SEQ ID NO:34) and predicted single letter code amino acid (SEQ ID NO:35) sequence are shown. The putative membrane spanning domain, deduced from hydrophilicity plots, is marked by a line. Potential N-glycosylation sites are shaded.

Please substitute the following paragraph for the paragraph beginning on page 11, line 28:

Figure 2: is an alignment of the predicted protein sequences for human (SEQ ID NO:35) and rat (SEQ ID NO:36) NAALAD-ase L. The amino acid sequences were aligned using the ClustalW alignment program (EMBL, Heidelberg Germany). Amino acid residues identical in both proteins are highlighted in black. Amino acid residues are numbered in the right hand margin.

Please substitute the following paragraph for the paragraph beginning on page 11, line 36:

Figure 3: is an illustration of alternative splicing of NAALAD-ase L. Amino acid sequence for NAALAD-ase L is shown. Sites at which putative DNA sequences are spliced out are marked by an arrow with the resulting (in-frame) amino acid deletions highlighted in bold italicised letters (SEQ ID NOS: 37, 38, 39, 40). Sites of putative intronic DNA insertion are marked by triangles, with the intronic DNA sequence shown above (SEQ ID NOS: 41, 43, 45). Resulting

changes to the amino acid sequence are highlighted in bold italicised letters (SEQ ID NOS: 42, 44, 46). Numbering of amino acid residues is to the right.

Please substitute the following paragraph for the paragraph beginning on page 12, line 11:

Figure 4: is a nucleotide (SEQ ID NO:47) and amino acid (SEQ ID NO:48) sequence of human NAALAD-ase II. The nucleotide and predicted one letter code amino acid sequence are shown. The putative membrane spanning domain, deduced from hydrophilicity plots, is marked by a line. Potential N-glycosylation sites are shaded.

Please substitute the following paragraph for the paragraph beginning on page 12, line 18:

Figure 5: is a nucleotide sequence and amino acid sequence of human NAALAD-ase IV. The nucleotide (SEQ ID NO:49) and predicted one letter code amino acid (SEQ ID NO:50) sequence are shown. The putative membrane spanning domain, deduced from hydrophilicity plots, is marked by a line. Potential N-glycosylation sites are shaded.

Please substitute the following paragraph for the paragraph beginning on page 12, line 25:

Figure 6: is an alignment of the predicted protein sequences for human NAALAD-ases I (SEQ ID NO:51), L (SEQ ID NO:35), II (SEQ ID NO:48) & IV (SEQ ID NO:50). The amino acid sequences were aligned using the ClustalW alignment program. Amino acid residues identical to all four proteins are shaded in black. Amino acid residues identical to three of the four proteins are shaded in grey. Amino acid residues are numbered to the right. A putative Zn²⁺ peptidase domain is highlighted between arrows and was identified by comparison to yeast and bacterial aminopeptidases. Putative residues involved in the catalytic site of the α/β hydrolase fold family of proteins are marked by three arrows (nucleophile-acid-base).

Please substitute the following paragraph for the paragraph beginning on page 13, line 8:

Figure 8: is an alignment of the NAALAD-ase peptidase domains with related peptidases (SEQ ID NOS:56-59). Amino acid sequences were aligned using the standard settings of CLUSTALW

alignment program. Similar amino acid residues conserved in proteins are shaded in black. Similar amino acid residues conserved in 80% of the proteins are shaded in dark grey. Similar amino acid residues conserved in 60-79% of the proteins are shaded in light grey. Amino acid residues are numbered to the right. Putative residues involved in zinc binding are marked by asterisks. The base residue thought to be important in catalysis is marked by an arrow. Sequence names other than NAALAD-ases correspond to sequence accession numbers in Swiss-Prot and SPTREMBL; Ape 3 yeast, *Saccharomyces cerevisiae* aminopeptidase Y (SEQ ID NO:52); P96152, *Vibrio cholerae* aminopeptidase (SEQ ID NO:53); .Ampx vibpr, *Aeromonas proteolyticus* aminopeptidase (SEQ ID NO:54), Application strgr, *Streptomyces griseus* aminopeptidase (SEQ ID NO:55). Putative residues involved in zinc binding are marked by asterisks. General base residue thought to be important in catalysis is marked by an arrow.

Please substitute the following paragraph for the paragraph beginning on page 18, line 36:

Cloning of NAALAD-ase I by PCR. Sequence data from human NAALAD-ase I (Accession no. M99487) was used to design primers to amplify the complete coding sequence of NAALAD-ase I by PCR. Primers used were NAALD1S2 (BamHI) = 5' -CCC GGA TCC GAG ATG TGG ATT CTC CTT CAC GAA AC -3' (SEQ ID NO:1) and NAALD1AS2 (XhoI) = 5' –CCC CTC GAG TTA GGC TAC TTC ACT CAA AGT CTC TGC -3' (SEQ ID NO:2) (restriction sites to be introduced are underlined). PCR amplification was performed using Human Marathon-Ready™ cDNA from prostate in a total reaction volume of 50 µl, containing 1X Expand Long Template™ PCR buffer 2, 0.2 mM of each dNTP, 0.2 µM each of oligonucleotide primers NAALD1S[1]2 (BamHI) and NAALD1AS[1]2 (XhoI), 1 µl of Marathon-Ready™ cDNA and 2.5 U of Expand Long Template PCR mix. Samples were pre-heated at 94°C for 5 min before addition of enzyme. Cycling was for 45 s at 94°C, 1 min at 55°C and 1 min 48 s at 68°C for 30 cycles, with a final step of 7 min at 72°C. PCR products were analyzed on a 1% agarose gel (wt/vol) in 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA (sodium salt), pH 8.3) and the most prominent DNA band was excised from the gel and purified with the Qiaquick gel extraction kit (Qiagen GmbH, Düsseldorf, Germany). The resulting 2303 base pair (bp) fragment was cloned into the plasmid vector pCR2.1 using the original TA cloning kit according to the manufacturer's instructions. Approximately 20 ng of purified fragment was ligated to 50

ng of pCR2.1 plasmid DNA with 4 U T4 DNA ligase in a total volume of 10 μ l. Ligations were incubated overnight at 14°C. 2 μ l of the ligation reaction was transformed into TOP10F' competent cells using heat-shock transformation and plated on 2x YT/ampicillin plates supplemented with IPTG and X-gal for blue-white screening. Colony screening was performed on 10 white colonies, from which plasmid DNA was prepared using the Qiagen plasmid mini DNA purification kit, and then digested with BamHI and XhoI. Four clones containing an insert of the appropriate size were sequenced fully. All the clones had at least one mis-sense PCR error. Clone 10.0 with a single PCR error at position 1183 was used as the template for a site directed mutagenesis (SDM) reaction using the QuickChange™ SDM Kit. Reactions were carried out according to the manufacturer's instructions. Primers designed for the amplification reactions were NAALD1-SDM-S1 = 5' -CCC TCA GAG TGG AGC AGC TGT TGT TCA TGA AAT TGT GAG G -3' (SEQ ID NO:3) and NAALD1-SDM-AS1 = 5' -CCT CAC AAT TTC ATG AAC AAC AGC TGC TCC ACT CTG AGG G -3' (SEQ ID NO:4). Three white clones from the SDM transformations were screened. Plasmid DNA was prepared using the Qiagen plasmid mini DNA purification kit, digested with BamHI and XhoI and sequenced around the mutation site. A single clone (cl.2.0) was sequenced fully on both strands to confirm the complete correct NAALAD-ase I sequence.

Please substitute the following paragraph for the paragraph beginning on page 20, line 18:

Cloning of NAALAD-ase L by PCR and 5' rapid amplification of cDNA ends (RACE) PCR. Sequence data from partial human NAALAD-ase L (GenBank Accession no. AF10141) was used to design primers to amplify the 3' end of NAALAD-ase L by PCR. Primers used were NAALD2S1 = 5' -GTT CTT CAA CAA GCT GCA GGA GCG -3' (SEQ ID NO:5) and NAALD2AS1 (XhoI) = 5' -CCC CTC GAG CCG GAG TAA AGG GAG GGC TGA AG -3' (SEQ ID NO:6). Human Marathon-Ready™ cDNA from brain, fetal brain, prostate, small intestine, colon were used in the amplification reactions. First round PCR amplification was performed in a total reaction volume of 50 μ l containing 1X Expand High Fidelity™ PCR buffer, 0.2 mM of each dNTP, 0.2 μ M each of oligonucleotide primers NAALD2S1 and NAALD2AS1, 1 μ l of Marathon-Ready™ cDNA and 2.5 U of Expand High Fidelity PCR mix. Samples were preheated at 94°C for 5 min before addition of the enzyme. Cycling was for 45 s at 94°C, 1 min

at 58°C and 1 min at 72°C for 30 cycles, with a final step of 7 min at 72°C. PCR products were analyzed on a 1% agarose gel in 1X TAE buffer. Second round PCR amplification was performed with nested primers NAALD2S2 = 5' -GGC GAC CTG AGC ATC TAC GAC AAC -3' (SEQ ID NO:7) and NAALD2AS2 (XhoI) = 5' -CCC CTC GAG TCC CCT CAG AGG TCA GCC ACA G -3' (SEQ ID NO:8). 1 µl of the fist round amplification reaction in a total volume of 50 µl containing 1X Expand High Fidelity™ PCR buffer, 0.2 mM of each dNTP, 0.2 uM each of oligonucleotide primers NAALD2S2 and NAALD2AS2 and 2.5 U of Expand High Fidelity PCR mix. Samples were preheated at 94°C for 5 min before addition of the enzyme. Cycling was for 45 s at 94°C, 1 min at 57°C and 1 min at 72°C for 30 cycles, with a final step of min at 72°C. PCR products were analyzed on a 1% agarose gel in 1X TAE buffer and the most prominent DNA bands were excised form the gel and purified with the Qiaquick gel extraction kit. The resulting fragments 600-800 bp were cloned into CR2.1 as described previously. Ligations were incubated overnight at 14°C and transformed into TOP10F' competent cells and plated on 2x YT/ampicillin plates supplemented with IPTG and X-gal for blue-white screening. Colony screening was performed on five white colonies from each transformation. Plasmid DNA was prepared from these colonies using the Qiagen plasmid mini DNA purification kit and then digested with EcoRI. Plasmids containing inserts of the appropriate size were end sequenced using vector primers and then the full sequencing on both strands of putative NAALAD-ase L clones was performed. Clone 6.9 derived from small intestine extended to the translation termination codon.

Please substitute the following paragraph for the paragraph beginning on page 21, line 32:

To obtain unknown 5' coding sequence for human NAALAD-ase L, two anti-sense primers were designed for 5' rapid amplification of cDNA ends (5' RACE). The primers were NAALD2AS3 = 5' -GCC AGC ACC CAG AGA ACC CAA G -3' (SEQ ID NO:9) and NAALD2AS4 = 5' -GCT GCG GTT GAA GTA CCG GAT C -3' (SEQ ID NO:10). Human Marathon-Ready™ cDNA from brain, fetal brain, prostate, small intestine and colon were used for the 5' RACE according to the manufacturer's instructions. The Marathon-Ready™ cDNA was prepared using oligo-dT priming and a Marathon cDNA adaptor (including two different adaptor-primer annealing sites) ligated to the 5' end of the cDNA. Adaptor-primer AP1 (5' -CCA TCC TAA

TAC GAC TCA CTA TAG GGC -3', SEQ ID NO:11) and nested adaptor-primer AP2 (5' -ACT CAC TAT AGG GCT CGA GCG GC -3', SEQ ID NO:12) were included in the kit. First round PCR amplification was performed in a total reaction volume of 50 µl containing 1X Expand High Fidelity™ PCR buffer, 0.2 mM of each dNTP, 0.2 µM each of oligonucleotide primers AP1 and NAALD2AS3, 5 µl of Marathon-Ready™ cDNA and 2.5 U of Expand High Fidelity PCR mix. Samples were preheated at 94°C for 5 min before addition of the enzyme. Cycling was for 30 s at 94°C, 30 s at 58°C and 2 min at 72°C for 30 cycles, with a final step of 7 min at 72°C. PCR products were analyzed on a 1% agarose gel in 1X TAE buffer. Second round 5' RACE was performed using 1 µl of the first round amplification reaction in a total volume of 50 µl containing 1X Expand High Fidelity™ PCR buffer, 0.2 mM of each dNTP, 0.2 µM each of oligonucleotide primers AP2 and NAALD2AS4 and 2.5 U of Expand High Fidelity PCR mix. Samples were preheated at 94°C for 5 min before addition of the enzyme. Cycling was for 30 s at 94°C, 30 s at 57°C and 1 min at 72°C for 30 cycles, with a final step of 7 min at 72°C. PCR products were excised from the gel and cloned into the vector pCR2.1 as described earlier. Colony screening by PCR was performed on 60 white colonies in 45 µl PCR mix containing 1X PCR buffer with MgCL₂, 0.2 mM dNTP, 0.5 µM each of vector primer M13FOR (5' -TGT AAA ACG ACG GCC AGT -3', SEQ ID NO:13) and M13REV (5' -CAG GAA ACA GCT ATG ACC -3', SEQ ID NO:14) and 0.35 U of super Taq DNA polymerase. Colonies were picked from the plates, inoculated into 100 µl LB medium supplemented with 100 µg/ml of ampicillin and incubated for 1 hr at 37°C. 5 µl of the incubated culture was then added to 45 µl PCR mix. PCR was performed for 30 cycles (45 s at 95°C, 1 min at 48°C and 50 s at 72°C). 20 µl of the PCR reactions was analyzed on 1% agarose gel in 1x TAE buffer. Clones containing inserts were grown overnight in 3 ml LB medium supplemented with 100 µg/ml of ampicillin and plasmid DNA was prepared using the Qiagen plasmid mini DNA purification kit. From the 51 clones sequenced one clone 4.10 contained 258 bp of sequence 5' to NAALD2AS4 of which 70 bp were novel.

Please substitute the following paragraph for the paragraph beginning on page 23, line 12:

The 5' RACE clone (cl. 4.10) and 3' PCR clone (cl. 6.9) were both digested with BamHI. The digested material was run on 1% agarose gel in 1X TAE buffer. Two bands were excised, a 336

bp band from cl. 4.10 containing all additional 5' RACE DNA sequences and a ~4700 bp fragment containing the remaining 3' NAALAD-ase L and vector sequence from cl. 6.9. The gel slices were purified with the Qiaquick gel extraction kit. The larger of the two fragments was dephosphorylated with 1.5 U calf of intestinal alkaline phosphatase for 1 hr at 37°C and then heat inactivated for 20 min at 75°C, in order to prevent the self ligation of the fragment with itself. Ligations were performed as described previously and 2 µl of the reaction mixture transformed into 35 µl DH10b electrocompetent cells by electroporation (single pulse; 2500V, 25 µF 201 W, 5 ms). The electroporated sample was added to 1 ml SOC media and incubated for 1 hr at 37°C before 100 µl of the culture was plated on to 2 x YT/ampicillin plates. Colonies were picked the following day, plasmid DNA prepared and tested by restriction digest. A single clone (cl. 2.0) was fully sequenced on both strands and found to contain the complete 3' coding sequence and the additional sequence from the first 5' RACE reactions. To obtain additional 5' coding sequence for the human NAALAD-ase L, two new anti-sense primers were synthesised corresponding to sequences form Incyte clone number 4190746. Primers used were NAALD2AS5 = 5' – CTG CAG CTT GTT GAA CTC TTC TGT G – 3' (SEQ ID NO:15) and NAALD2AS6 = 5' CAA ACA CGA TTG ATC TGC GAG GAC – 3' (SEQ ID NO:16). Human Marathon-Ready™ cDNA from brain, fetal brain, prostate, small intestine, colon and heart were used for the 5' rapid amplification of cDNA ends (5' RACE) according to the manufacturer's instructions. First round PCR amplification was performed in a total reaction volume of 50 µl containing 1X Expand High Fidelity™ PCR buffer, 0.2 mM of each dNTP, 0.2 µM each of oligonucleotide primers AP1 and NAALD2AS5, 5µl of Marathon-Ready™ cDNA and 2.5 U of Expand High Fidelity PCR mix. Samples were preheated at 94°C for 5 min before addition of the enzyme. Cycling was for 30 s at 94°C, 30 s at 58°C and 1 min at 72°C for 30 cycles, with a final step of 7 min at 72°C. PCR products were analysed on a 1% agarose gel in 1X TAE buffer (40 mM Tris-acetate, 1mM EDTA, pH 8.3). Second round PCR amplification was performed using 1 µl of the first round amplification reaction in a total volume of 50 MG containing 1X Expand High Fidelity™ PCR buffer, 0.2 mM of each dNTP, 0.2 µM each of oligonucleotide primers AP2 and NAALD2AS6 and 2.5 U of Expand High Fidelity PCR mix. Samples were preheated at 94°C for 5 min before addition of the enzyme. Cycling was for 30 s at 94°C and 1 min at 72°C. PCR products were analysed on a 1 % agarose gel in 1X TAE buffer and the 8 most prominent DNA bands were excised from the gel and purified with the Qiaquick gel

extraction kit. The resulting fragments (400-1300bp) were cloned into the plasmid vector pCR2.1 using the original TA cloning kit. Ligations and transformations were performed as described earlier before plating on 2x YT / ampicillin plates supplemented with IPTG and X-gel for blue-white screening. Colony screening was performed on five white colonies from each transformation. Plasmid DNA was prepared from these colonies using the Qiagen plasmid mini DNA kit and digested with EcoRI. Plasmids containing inserts of the appropriate size were end sequenced using vector primers and then the full sequence of putative NAALAD-ase L clones was determined using primer walking. The DNA sequence of five clones, from a small intestine cDNA, extended the coding sequence for NAALAD-ase L in the 5' direction beyond the putative translation start codon and included part of the 5' untranslated region. One of these clones one (cl. 2.2) was used for further experiments.

Please substitute the following paragraph for the paragraph beginning on page 25, line 15:

To construct a full length NAALAD-ase L clone, two new primer sets were designed to introduce a unique restriction site (Mun I) into the DNA sequence of NAALAD-ase L without resulting in a change in amino acid sequence or frame shift in the open reading frame (ORF). The first primer set was NAALD2S3 (EcoRV) = 5' – CGG ATA TCC GCA GGA TGC AGT GGA CGA AG – 3' (SEQ ID NO:17) and NAALD2AS8 (MunI) – 5' – CAA ACA CAA TTG ATC TGC GAG GAC GC – 3' (SEQ ID NO:18) and the second primer set was NAALD2S8 (MunI) = 5' – GCG TCC TCG CAG ATC AAT TGT GTT TG – 3' (SEQ ID NO:19) and NAALD2AS1 (XhoI). PCR amplification was performed on 1 cl. 2.0 plasmid DNA with primers NAALD2S3 (EcoRV) and NAALD2AS8 (MunI) or on 1 μ l cl. 2.2 plasmid DNA with primers NAALD2S3 (MunI) AND NAALD2AS1 (XhoI). Total reaction volumes were as previously described. Samples were preheated at 94°C for 5 min before addition of the Expand High Fidelity enzyme. Cycling was for 45 s at 95°C, 1 min at 57°C and 1 min at 72°C for 30 cycles; with a final step of 7 min at 72°C. PCR products were analysed on a 1% agarose gel in 1X TAE buffer and TA cloned into pCR2.1 as previously described.

Please substitute the following paragraph for the paragraph beginning on page 26, line 11:

Cloning of NAALAD-ase II by PCR and 5' rapid amplification of cDNA ends (RACE) PCR. Sequencing results from Incyte clone 3608639 suggested that this clone contained DNA sequence spanning the complete coding sequence, 2220 bp in size, of a putative NAALAD-ase like molecule (NAALAD-ase II) that had similar sequence to NAALAD-ase I and L. To confirm that there was no possible initiation codon upstream of the initiation codon already determined 5' RACE PCR was performed. Two anti-sense primers were designed for 5' RACE based on the sequence derived from the clone 3608639, NAALD3AS1 – 5' – CTT TGA TGA TAG CGC ACA GAA GTG G – 3' (SEQ ID NO:20) and NAALD3AS2 – 5' GGA AAG ATG CCA GCG CAG GAC 03' (SEQ ID NO:21). Human Marathon-Ready™ cDNA from brain, foetal brain, prostate, small intestine and colon were used for the 5' RACE according to the manufacturer's instructions. First round PCR amplification was performed in a total reaction volume of 50 µl containing 1X Expand High Fidelity™ PCR buffer, 0.2 mM of each dNTP, 0.2 µM each of oligonucleotide primers AP1 and NAALD3AS1, 5 µl of Marathon-Ready™ cDNA and 2.5 U of Expand High Fidelity PCR mix. Samples were preheated at 94°C for 5 min before addition of the enzyme. Cycling was for 30 s at 94°C, 30 s at 58°C and 2 min at 72°C for 30 cycles, with a final step of 7 min at 72°C. Second round 5' RACE was performed using 1 µl of the first round amplification reaction in a total volume of 50 µl containing 1X Expand High Fidelity™ PCR buffer, 0.2 mM of each dNTP, 0.2 µM each of oligonucleotide primers AP2 and NAALD3AS2 and 2.5 U of Expand High Fidelity PCR mix. Samples were preheated at 94°C for 5 min before addition of the enzyme. Cycling was for 30 s at 94°C, 30 s at 57°C and 1 min at 72°C for 30 cycles, with a final step of 7 min at 72°C. PCR products were analysed on a 1% agarose gel in 1X TAE buffer and the most prominent DNA bands were excised from the gel and purified with the Qiaquick gel extraction kit. The resulting fragments (250 – 600 bp) were cloned into the plasmid pCR2.1 as described previously. 32 white colonies were grown overnight in 3 ml LB medium supplemented with 100 µg/ml of ampicillin and plasmid DNA was prepared using the Qiagen plasmid mini DNA purification kit. No upstream initiation codon was identified from any of the 32 clones analysed.

Please substitute the following paragraph for the paragraph beginning on page 27, line 20:

Cloning of NAALAD-ase IV by PCR.

Sequencing results from Incyte clone 2615389 revealed that this clone contained partial coding sequence and 3' UTR, of another putative NAALAD-ase like molecule (NAALAD-ase IV) that was related in sequence to NAALAD-ase I, L and II. The DNA sequence obtained was used in a BLAST search on the Incyte LifeSeq™ EST database. One contig (2519841) was assembled from 150 overlapping Incyte EST sequences that spanned 1881 bp and contained a coding region of 1419 bp. The sequence data from human NAALAD-ase IV contig 2519841 was used to design primers to amplify the complete coding sequence of by PCR. Primers used were NAALD4SP2 = 5' CGT CAG AGC CGC CCT ATC AGA TTA TC – 3' (SEQ ID NO:22) and NAALD4AP4 = 5' – GAG GAG TTT TCC AAA GTT GCA GAC CC – 3' (SEQ ID NO:23). PCR amplification was performed using a human hippocampal cDNA in a total reaction volume of 50 µl, containing 1X Expand High Fidelity™ PCR buffer, 0.2 mM of each dNTP, 0.2 µM each of oligonucleotide primers NAALD4SP2 and NAALD4APA4, 1µl of cDNA and 2.5 U of Expand High Fidelity™ PCT mix. Samples were pre-heated at 95°C for 5 min before addition of enzyme. Cycling was for 45 s at 94°C, 1 min at 58°C and 35 s at 72°C for 30 cycles, with a final step of 7 min at 72°C. PCR products were analysed on a 1% agarose gel in 1X TAE buffer and the most prominent DNA band was excised from the gel and purified with the Qiaquick gel extraction kit. The resulting 1544 bp DNA fragment was sub-cloned into the plasmid vector pCR2.1-TOPO using the TA TOPO cloning it according to the manufacturer's instructions. Approximately 10 ng of purified fragment was ligated to 10 ng of pCR2.1-TOPO plasmid DNA. Ligations were incubated for 5 min at 25°C. Transformations into TOP10F' competent cells and colony screening was performed as previously described. Three clones containing an insert of the correct size were sequenced fully and two clones were found to contain no PCR errors (cl. 28.0 and cl. 1.0).

Please substitute the following paragraph for the paragraph beginning on page 33, line 3:
Gene Expression of NAALAD-ase I, L, II and IV by RT-PCR analysis.

Oligonucleotide primers designed for the specific amplification of a PCR fragment for each NAALD-ase; NAALD-ase I primers were NAALAD1S3 5' – GGG AAA CAA ACA AAT TCA GCG GC – 3' (SEQ ID NO:24) and NAALD1AS3 5' GTC AAA GTC CTG GAG TCT CTC ACT GAA C – 3' (SEQ ID NO:25) yielding a 341 bp product, NAALAD-ase L primers were NAALD2S7 5' GAC CGG AGC AAG ACT TCA GCC AG – 3' (SEQ ID NO:26) and

NAALD2AS7 5' – GTG TTG ATA TGC GTT GGC CCA AG – 3' (SEQ ID NO:27) yielding a 330 bp product, NAALAD-ase II primers were NAALD3S4 5' CAC TAA GAA TAA GAA AAC AGA TAA GTA CAG C-3' (SEQ ID NO:28) and NAALD3AS4 5' – GAT CAA CTT GTA TAA GTC GTT TAT GAA AAT CTG – 3' (SEQ ID NO:29) yielding a 353 bp product and NAALAD-ase IV primers were NAALD3S1 5' – GCA GAA GAA CAA GGT GGA GTT GGT G – 3' (SEQ ID NO:30) and NAALD4AS1 5' – GCT TTG GAT CCA TGA CAG TCA TGG – 3' (SEQ ID NO:31) yielding a 336 bp product. Each primer set for each NAALAD-ase was tested for its ability to specifically amplify that NAALAD-ase and not to cross react in amplification reactions with the other three forms. PCR amplifications for human GAPDH were performed on the same cDNA samples as positive controls using GAPDH specific primers 5' - TGA AGG TCG GAG TCA ACG GAT TTG GT – 3' (SEQ ID NO:32) (sense primer) and 5' - CAT GTG GGC CAT GAG GTC CAC CAC – 3' (SEQ ID NO:33) (anti-sense primer), yielding a 1000 bp fragment. These primers sets were used for PCR amplifications on human multiple tissue cDNA (MTCTM) panels normalised to the mRNA expression levels of six different housekeeping genes. Human cDNAs from 15 brain regions were also prepared from mRNA and normalised to the mRNA expression levels of three different housekeeping genes, GAPDH, clathrin and actin. Brain area mRNA was prepared starting from carefully dissected tissue samples, using the FastTrackR 2.0 kit (Invitrogen BV, Netherlands) according to the manufacturer's instructions. 1 μ g of poly (A) + RNA was reverse transcribed using oligo (dT) 15 as a primer and 50 U of Exapnd™ Reverse Transcriptase (Boehringer Mannheim, Germany) according to the manufacturer's instructions. Finally, normalised cDNA was prepared in a similar manner from 3 transformed prostate tumour cell lines and a prostate tumour. PCR reactions with NAALAD-ase or GAPDH specific primers were performed on 2 or 5 μ l of cDNA. PCR reactions were performed in a total volume of 50 μ l in 1X Advantage PCR buffer, 0.2 mM dNTP and 0.5 μ M of each PCR primer and 1 μ l Advantage Taq polymerase mix (95°C – 30 sec, 68°C – 1 min 30 s for 35 cycles). Upon completion of 25 cycles the PCR machine was paused at 80C, reaction tubes were removed and 15 μ l were removed from each PCR tube. Tubes were then returned to the machine and the cycling method continued. Aliquots were removed in a similar manner after 30 and 35 cycles. Each sample taken was analysed by 1.0% agarose gel electrophoresis as previously described and images of the ethidium bromide stained gels were obtained using an Eagle Eye II Video system (Stratagene, La Jolla, CA, USA).